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Vitamin C is dispensable for oxygen sensing *in vivo*

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Short Title:
ROLE OF VITAMIN C IN OXYGEN SENSING

Regular Article
RED CELLS, IRON, AND ERYTHROPOIESIS

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Abstract

Prolyl-4-hydroxylation is necessary for proper structural assembly of collagens and oxygen-dependent protein stability of hypoxia-inducible transcription factors (HIFs). *In vitro* function of HIF prolyl-4-hydroxylase domain (PHD) enzymes requires oxygen and 2-oxoglutarate as co-substrates with iron (II) and vitamin C serving as co-factors. While vitamin C deficiency is known to cause the collagen-disassembly disease scurvy, it is unclear whether cellular oxygen sensing is similarly affected. Here we report that vitamin C deprived *Gulo*$^{-/-}$ knock-out mice show normal HIF-dependent gene expression. The systemic response of *Gulo*$^{-/-}$ animals to inspiratory hypoxia, as measured by plasma erythropoietin levels, was similar to animals supplemented with vitamin C. Hypoxic HIF induction was also essentially normal under serum- and vitamin C-free cell culture conditions, suggesting that vitamin C is not required for oxygen sensing *in vivo*. Glutathione was found to fully substitute for vitamin C requirement of all three PHD isoforms *in vitro*. Consistently, glutathione also reduced HIF-1α protein levels, transactivation activity and endogenous target gene expression in cells exposed to $CoCl_2$. A C201S mutation in PHD2 increased basal hydroxylation rates and conferred resistance to oxidative damage *in vitro*, suggesting that this surface accessible PHD2 cysteine residue is a target of antioxidative protection by vitamin C and glutathione.

Introduction

Oxygen is essential for a number of physiological processes, particularly for cellular respiration and energy metabolism. On the molecular level, response to hypoxia is mediated by hypoxia-inducible transcription factors termed HIFs. Under continuous oxygen supply, two distinct prolyl residues within the oxygen-dependent degradation domain (ODD) of HIFα subunits are hydroxylated by prolyl-4-hydroxylase domain-containing enzymes (PHDs). Hydroxy-HIFα is recognized by the von Hippel-Lindau tumor suppressor protein (pVHL) and subsequently targeted for proteasomal degradation [1,2]. When oxygen is limited, PHD activity ceases, non-hydroxylated HIFα is stabilized and heterodimerizes with the HIFβ subunit to activate expression of numerous target genes [3]. Moreover, an asparaginyl hydroxylase termed factor inhibiting HIF (FIH) hydroxylates a C-terminal Asn residue of HIF-α subunits in an oxygen-dependent manner, thereby regulation co-factor recruitment and HIF's transcriptional activity [4].

Three PHD isoforms have been characterized so far, termed PHD1, PHD2 and PHD3, which differ in size, subcellular localization and tissue distribution [5]. PHD2 is the most ubiquitously expressed isoform, responsible for the normoxic control of HIFα [6]. Accordingly, genetic ablation of PHD2 but not PHD1 or PHD3 results in embryonic lethality in mice [7]. Suggesting a fundamental role in the hematopoietic and circulatory systems, somatic inactivation of PHD2 leads to increased erythropoiesis and angiogenesis as a result of HIFα stabilization followed by activation of its target genes, including erythropoietin (EPO) and vascular endothelial growth factor (VEGF) [8]. Knock-out of either PHD1 or PHD3 had no effect on hematological parameters. However, combined PHD1/PHD3 knock-out animals showed a slight increase in hematocrit, hemoglobin and red blood cell counts [9]. Clinical data on patients with erythrocytosis revealed P317R or R371H mutations in the gene encoding for PHD2, altering the hydroxylation efficiency of the mutant protein [10,11]. A third point mutation in PHD2 (H374R) was found in a patient suffering from erythrocytosis and paraganglioma [12]. These case reports emphasize the critical role of PHD2 in regulating erythropoiesis and maintaining red blood cell homeostasis also in humans.

PHDs belong to a larger superfamily of 2-oxoglutarate and Fe (II)-dependent di-oxygenases. Similar to collagen prolyl-4-hydroxylase (C-P4H, EC 1.14.11.2), PHDs require molecular oxygen and 2-oxoglutarate as co-substrates, as well as ferrous iron and probably

vitamin C as co-factors for enzymatic activity [13]. K_m-values of PHDs for oxygen are strikingly higher than those of other prolyl-4-hydroxylases [13]. The relatively low oxygen affinity is essential for effective oxygen sensing, since even small changes in oxygen partial pressure can influence hydroxylation activity [13-15].

In a previous study we reported on the dose-dependent regulation of the *in vitro* activity of all three PHD isoforms by their essential co-substrates and co-factors, including vitamin C [16]. Primates, including humans, lost the ability to *de novo* synthesize vitamin C and thus depend on dietary vitamin C intake. Since ascorbate is an essential co-factor for C-P4Hs which hydroxylate proline residues to stabilize the collagen triple helix structure, persistent ascorbate deficiency results in disassembly of connective tissue structures, a common symptom of the nowadays rare disease scurvy [17]. With K_m-values ranging from 140-180 µM, the requirement of PHDs for vitamin C *in vitro* is only two fold lower than for C-P4H, suggesting that also HIF hydroxylases could well be affected by vitamin C malnutrition [13]. Mice lacking a functional *Gulo* gene have been described as a model to study vitamin C deficiency [18]. *Gulo* encodes for L-gulono-1,4-lactone-oxidase (GLO, EC 1.1.3.8), a key enzyme involved in the final step of L-ascorbic acid (vitamin C) biosynthesis. Dietary vitamin C deprivation leads to body weight loss, anemia, aortic wall damage and internal hemorrhages in these mice [18].

While the interaction between the target prolyl residue, molecular oxygen, 2-oxoglutarate and iron during the reaction cycle in the active center of PHDs has been described in detail, the apparently inevitable presence of vitamin C for the *in vitro* function of the PHDs remains elusive [19,20]. Due to its antioxidative properties, vitamin C might maintain ferrous iron in the reduced state. Given the enzymatic relationship between HIFα and collagen prolyl-4-hydroxylases, we set out to investigate the effect of dietary vitamin C on the regulation of the PHD-HIF oxygen sensing pathway in *Gulo*$^{-/-}$ mice under normoxaemic and hypoxaemic conditions.

Methods

Cell culture. HeLa human cervix carcinoma cells were adapted to Ham´s Nutrient Mixture F12 (Sigma), free of ascorbate and FCS, containing the following supplements: EGF (50 ng/ml, Sigma), insulin (5 µg/ml, Sigma), apo-transferrin (5 µg/ml, Sigma), hydrocortisone (100 nM, Sigma) buffered with 15 mM Hepes (pH 7.4) as well as 100 IU/ml penicillin and 100 µg/ml streptomycin. HepG2 human hepatoma cells stably transfected with a HIF-dependent firefly luciferase reporter gene termed HRG1 have been described before [21]. If not indicated otherwise, all cells were maintained in Dulbecco's Modified Eagle Medium with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cell number and viability was determined using a ViCell Counter (Beckman).

HIF transactivation activity. 5×10^5 HeLa cells were co-transfected with 500 ng of the HIF-dependent pH3SVL reporter vector containing a total of 6 HIF binding sites derived from regulatory elements of the *transferrin* gene [22] and 40 ng of pRL-CMV *Renilla* luciferase expression plasmid (Promega) essentially as described before [14]. 24 hours post transfection, cells were split and exposed to graded oxygen concentrations (21%-0.2% oxygen) for 24 hours using cross-calibrated oxygen-controlled CO_2 incubators (Binder CB 150). Stably transfected HRG1 HIF reporter cells were adapted to 1% FCS overnight and treated with 50 µM desferrioxamine mesylate (Dfx, Sigma) or 100 µM $CoCl_2$ and 1-10 mM reduced glutathione (GSH, 250 mM stock solution adjusted to pH 7.0) or 0.2-2 mM ascorbate for 24 hours. For hypoxic experiments, cells were grown under 2% O_2 for 24 hours and treated with GSH or ascorbate. HRG1 cells were transfected with pRL-SV40 *Renilla* luciferase to control for non-HIF mediated effects of ascorbate and GSH on the heterologous SV40 minimal promoter present in both constructs. Cells were lysed using passive lysis buffer and luciferase activities were determined according to the manufacturer's instructions (Promega) using a 96-well luminometer (Berthold). Data are expressed as relative luciferase activities per total cellular protein of experiments performed in triplicates by calculating the ratio of *firefly/renilla* activities per well.

Expression and purification of recombinant PHD enzymes. Recombinant PHD proteins were expressed and purified as glutathione-S-transferase (GST)-fusion proteins from baculovirus-infected Sf9 insect cells as described earlier [14]. Untagged enzyme preparations were

obtained by introducing a PreScission protease cleavage site between the GST-tag and the PHD open reading frame. A C201S point mutation was introduced into the human PHD2 expression plasmid by site-directed mutagenesis (Stratagene). Untagged PHD2 was expressed in Sf9 cells and purified by conventional ion-exchange chromatography (kind gift of Dr. Felix Oehme, Bayer Healthcare, Wuppertal, Germany). Purity of the enzyme preparations was analyzed by SDS-PAGE followed by Coomassie staining or immunoblotting.

Prolyl-4-hydroxylation assay. Activity of recombinant PHD enzymes was measured by a microtiter plate-based peptide hydroxylation assay as described before [23]. Briefly, recombinant PHDs were used to hydroxylate a biotinylated peptide derived from HIF-1α (amino acid residues 556 to 574) coupled to streptavidin-coated 96-well plates. Hydroxylation reaction was performed for 1 hour at room temperature in the presence of 10 μM $FeSO_4$, 0.5 mM 2-oxoglutarate and 2 mM ascorbate in 20 mM Tris-HCl pH 7.5, 5 mM KCl and 1.5 mM $MgCl_2$. Hydroxylated peptides were detected by recombinant, thioredoxin-tagged von Hippel-Lindau/elongin B/elongin C (VBC) complex. Reactions were stopped by removing the reaction mix and adding 1 mM H_2O_2. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies and secondary HRP-conjugated anti-rabbit antibodies (Sigma) using the 3,3,5,5-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding 2 M H_2SO_4 and absorbance was determined at 450 nm in a microplate reader. Background values as determined by using a mutant HIF-1α (P564A) peptide were subtracted for each experiment.

Ascorbate determination. Ascorbate content of *in vitro* hydroxylation assay samples was quantified by HPLC as described before [24]. Briefly, a 10-fold dilution of the enzyme reaction mix containing 2 mM ascorbate was analyzed before and after 1 hour of hydroxylation reaction. Following dilution in the mobile phase (60 mM phosphoric acid, pH 3.1), a 20 μl sample was injected onto a Nucleosil C18 column and eluted applying an acetonitrile gradient (0-60%). Ascorbate elution was monitored at 254 nm, corresponding to 96% absorbance of ascorbate and only 4% of dehydroascorbate [25]. Chromatograms and standard curve of pure ascorbate ranging from 25-200 μM were used to calculate the content of ascorbate in study samples (Supplementary Fig. 2). Ascorbate levels in plasma samples of mice were determined by HPLC (Swiss Vitamin Institute, Epalinges, Switzerland).

Immunoblot analyses. Total soluble cellular proteins were extracted with a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma). Protein concentration was measured by the method of Bradford and 60-70 µg of cellular protein was subjected to immunoblot analyses. Membranes were probed using the following dilutions of mouse monoclonal (mAb) or rabbit polyclonal antibodies: mAb anti-HIF-1α (BD Transduction Laboratories, 1:1000), mAb anti-CA9 (M75, kindly provided by S. Pastorekova, Bratislava, Slovak Republic), mAb anti-β-actin (Sigma, 1:5000), rabbit anti-PHD1 (Genway, 1:2000), rabbit anti-PHD2 (Novus, 1:1000) or rabbit anti-PHD3 (Novus, 1:1000), followed by secondary HRP-conjugated antibodies (all Pierce, 1:2000).

mRNA quantification. Total RNA purification and mRNA determination by real-time PCR has been described before [14]. Transcript levels of the HIF-dependent and -independent genes were quantified by reverse transcription (RT) quantitative (q) PCR using SybrGreen qPCR reagent kit (Sigma) in combination with an MX3000P light cycler (Stratagene). Initial copy number of each sample was calculated by comparison with serial dilutions of a calibrated standard. For mouse tissues, ribosomal protein S12 mRNA was used as a housekeeping gene while ribosomal protein L28 mRNA served as control for samples from human cell lines. Primer sequences are given in *Supplementary Table 1*.

Oxyblot detection of protein oxidation. PHD2 carbonylation was determined with a protein oxidation detection kit (Oxyblot, Millipore). Briefly, 50 ng/µl of recombinant PHD2 was exposed to either 100 µM $CoCl_2$, 10 µM $FeSO_4$, 2 mM ascorbate, 0.5 mM 2-oxoglutarate or 1 mM H_2O_2 in the presence of 400 nM wild-type or P564A mutant HIF-1α peptide in 20 mM Tris-Cl, 5 mM KCl and 1.5 mM $MgCl_2$ for 1 hour at room temperature. Five µl of the reaction mix were mixed with 5 µl 12% SDS and carbonyl groups were derivatized with 10 mM 2,4-dinitrophenylhydrazine (DNPH) for 15 minutes. DNP groups were detected by immunoblotting using rabbit anti-DNP antibodies (1:150) followed by secondary goat anti-rabbit HRP-conjugated antibodies (1:300). For loading controls, PHD2 was detected using rabbit anti-PHD2 antibodies (Novus).

Animal studies. *Gulo*$^{-/-}$ mice were maintained on vitamin C–supplemented water containing 0.33 g/L of L-ascorbic acid and 0.01 mM EDTA as described earlier [18]. At 3 months of age, ascorbic acid supplement was withdrawn from six *Gulo*$^{-/-}$ males while continued for the control males. After 5 weeks, mice were killed with an overdose of 2,2,2-tribromoethanol, and tissues were collected and stored in RNAlater (Applied Biosystems Inc, Foster City, CA) at -20^{o}C until use. For hypoxia studies, 22 *Gulo*$^{-/-}$ males with an average age of 16 weeks were allocated to four groups so that no significant changes were observed in mean body weight and age of the animals at the beginning of the experiment. The body weight was determined every second day. After 36 days of ascorbate withdrawal, mice were exposed to 8% oxygen for 24 hours in a hypoxia tent (Coy Laboratory Products Inc.). Control animals were maintained at ambient oxygen concentration. Heparinized whole blood was collected from all mice by cardiac puncture after intraperitoneal injection anaesthesia using 4 mg/ml xylazine and 20 mg/ml ketamine at a dosage of 0.1 ml/20 g of body weight. Blood samples of hypoxic animals were collected inside the hypoxic tent. Animal experiments were conducted at two centers with the appropriate consent by the Institutional Animal Care and Use Committees of the University of North Carolina–Chapel Hill for breeding and normoxic gene expression studies or the Veterinary Office of the Canton Zürich (119/2010) for hypoxia studies, respectively.

Blood parameters and plasma EPO concentrations. Plasma EPO levels were measured by ELISA following the procedures recommended by the manufacturer (Quantikine, R&D Systems). EPO concentrations were determined by comparison to a calibrated recombinant mouse EPO standard. Hematologic parameters of mouse whole blood were analyzed by the Division of Hematology (University Hospital, Zürich, Switzerland).

Results

Ascorbate is not required for HIF induction by hypoxia in HeLa cells. HeLa cells have been described previously to grow in serum-free medium supplemented with hormones and growth factors [26].To achieve cell culture conditions avoiding ascorbate contamination derived from animal sera, Hela cells were adapted to a chemically defined medium free of ascorbate for at least 2 weeks. Control cells were grown in the same medium supplemented with 50 μM ascorbate. Both cell groups proliferated at the same rate with no differences noticed between ascorbate-free and supplemented cells (Fig. 1A). Despite its need for PHD activity *in vitro*, hypoxic HIF-1α protein accumulation was similar in ascorbate containing and deficient cells. However, a faint normoxic induction of HIF-1α could be observed in ascorbate-free cells only (Fig. 1B). Accordingly, cells transfected with a HIF-responsive reporter gene (pH3SVL) and subsequently exposed to graded oxygen concentrations (0.2, 1, 3 or 21% O_2, respectively) revealed similar induction levels of luciferase activity under both culture conditions (Fig. 1C).

GSH can substitute for vitamin C in the hydroxylation reaction catalyzed by PHDs. Because HeLa cells grown in a medium containing no ascorbate maintained hypoxic HIF-1α stabilization, we speculated that other antioxidants could compensate for vitamin C loss in these cells. Thus, a number of compounds with antioxidative properties were tested for their effects on PHD hydroxylation activity using a previously described *in vitro* hydroxylation assay [23]. Surprisingly, some compounds such as n-propyl gallate[16] and the superoxide dismutase mimetic Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) were potent inhibitors of PHD enzymes (Supplementary Fig. 1A). However, reduced L-γ-glutamyl-L-cysteinyl-glycine (glutathione; GSH) enhanced HIFα hydroxylation by all three PHDs. Since the recombinant enzyme preparations in the initial experiments were expressed and purified as glutathione-S-transferase (GST) fusion proteins, we could not exclude interference of the tested antioxidants, particularly GSH, with the GST tag. Therefore, a PreScission protease cleavage site was engineered in between the two fusion partners. Chimeric GST.PHD and tag-free PHD enzymes showed comparable hydroxylation activity (Supplementary Fig. 1B). GSH increased the activity of all three untagged PHD isoforms, even in the complete absence of ascorbate (Fig. 2A). Addition of GSH ("+5 mM" in Fig. 2B) to 2 mM ascorbate increased the reaction rate when compared with ascorbate alone (“control” in Fig. 2B), suggesting two independent reaction

modes of GSH, one replacing vitamin C and an additional one enhancing the reaction rate. Of note, only minor changes in ascorbate oxidation were found before and after one hour of PHD2-mediated substrate hydroxylation (Fig. 2C). The minor decrease in reduced ascorbate was most likely due to air-dependent oxidation rather than enzymatic consumption since it was independent of the presence of the hydroxyl-acceptor substrate (Fig. 2C, right panel). In conclusion, as shown previously for collagen P4H [27], ascorbate is not consumed during coupled PHD-catalyzed hydroxylation reactions.

GSH decreases HIF activity in $CoCl_2$-treated hepatoma cells. In cell culture models, Co (II) and Ni (II) have been shown to substantially decrease cellular ascorbate content by catalyzing ascorbate oxidation to DHA followed by irreversible hydrolysis to diketogulonate [28]. Interestingly, exogenous ascorbate administration completely blunted the Co (II) induced hypoxic response in lung epithelial cells [29]. To test if GSH could similarly compensate for reduced ascorbate levels after Co (II) stimulation, HIF transcriptional activity was further studied in HepG2 hepatoma cells stably transfected with a HIF-dependent luciferase reporter gene (HRG1 cells). We first determined the concentrations of $CoCl_2$ required to activate HIF-dependent reporter gene expression to a similar extent as exposure of the cells to 2% oxygen or the hypoxia mimicking iron chelator desferrioxamine (Dfx; Fig. 3A). Subsequently, HRG1 cells were treated with 100 µM $CoCl_2$ or 50 µM Dfx under 21% or 2% oxygen. Control cells were kept at ambient oxygen concentrations. All cells were co-treated with 1-10 mM GSH or 0.2-2 mM ascorbate. Indeed, ascorbate and GSH reduced HIF activity exclusively in $CoCl_2$-treated HRG1 cells (Fig. 3B). A substantial increase of HIF activation was noted particularly when cells were treated with 2 mM ascorbate, which might be explained by the pro-oxidative function ascorbate exerts if applied at high concentration (Fig. 3B, upper panel) [30]. In line with these observations, both ascorbate and GSH reduced the expression levels of the endogenous HIF target genes CA9 and NDRG1 [3] only in cells treated with $CoCl_2$, whereas 2 mM ascorbate enhanced hypoxic activation of both genes by almost 2-fold (Fig. 3C). To evaluate if these effects reflected differential activities of cellular PHD enzymes, HIF-1α protein accumulation was analyzed by immunoblotting. As expected, only $CoCl_2$-stabilized HIF-1α was down-regulated by co-treatment with ascorbate or GSH (Fig. 3D). Interestingly, 10 mM GSH was a more potent inhibitor of $CoCl_2$-induced HIF-1α stabilization than 2 mM ascorbate, a

concentration which showed saturated inhibition of the HIF-reporter in the same cell line (Fig. 3B, upper panel)

GSH protects PHD2 from metal-catalyzed oxidation. Enzymatic activity of the PHDs is sensitive to reactive oxygen species (ROS) and transition metal ions [31]. However, the mechanism(s) by which ROS or metal ions inhibit hydroxylase activity remained speculative. Besides its general antioxidative properties as radical scavenger, vitamin C actively interferes with the oxidation state of metal ions by serving as electron donor in a redox reaction. As such it largely differs from GSH which is a major cellular antioxidant protecting cysteinyl and methionyl residues in proteins from oxidative modifications. Both, $CoCl_2$ and H_2O_2 inhibited all three PHD isoforms *in vitro* with PHD2 being slightly more resistant to $CoCl_2$ (Supplementary Fig. 3). To directly determine protein oxidation by these compounds, carbonyl group formation in PHD2 was estimated by Oxyblot technology. As shown in Fig. 3E (left panel), 2 mM ascorbate and 10 μM $FeSO_4$ (as used in the standard reaction buffer for *in vitro* hydroxylation) substantially increased carbonylation of recombinant PHD2 during one hour of hydroxylation reaction. Surprisingly, $CoCl_2$ increased carbonylation of PHD2 whether ascorbate/$FeSO_4$ was present or not. Following addition of 5 mM GSH, oxidation of PHD2 by ascorbate/$FeSO_4$, $CoCl_2$ and H_2O_2 was markedly reduced. To examine whether PHD2 protein oxidation is coupled to its di-oxygenase activity, the reaction was performed in the presence of a mutant P564A HIF-1α peptide substrate. As shown in Fig. 3E (right panel), PHD2 protein oxidation was independent of the presence of a hydroxylation acceptor proline, providing evidence that protein oxidation is not caused by the hydroxylation reaction cycle.

Cysteine 201 affects PHD2 hydroxylation activity. Recently, cysteine residue C201 within the catalytic domain of PHD2 has been identified as a surface accessible, highly nucleophilic residue predominantly interacting with thiol compounds [32]. Moreover, C201 and C208 were proposed to provide an additional metal binding site in PHD2 [33]. Both, C201 and C208 are highly conserved among all three human and mouse PHD isoforms (Fig. 4A). To investigate the functional relevance of C201, recombinant PHD2, wild-type or C201S mutant, was purified from Sf9 cells. Surprisingly, the C201S mutation significantly ($p<0.0001$) increased the PHD2 reaction rate by 2.5-fold (Fig. 4B, upper panel). Equal concentrations of wild-type and

mutant PHD2 proteins were confirmed by Coomassie staining of the undiluted stock solutions and immunoblotting of the diluted assay solutions (Fig. 4B, lower panel). To further test the hypothesis that the C201S mutation might protect the PHD2 enzyme from oxidative damage, the effect of H_2O_2 on hydroxylation activity was measured. As shown in Fig. 4C, the half-maximal inhibitory concentration (IC_{50}) for H_2O_2 was roughly 5-fold higher for the C201S mutant compared with wild-type PHD2 (IC_{50} of 8.9×10^{-6} and 4.3×10^{-5} M H_2O_2 for wild-type and C201S mutant PHD2, respectively). We further tested if the H_2O_2 mediated loss of PHD2 activity could be rescued by sequential addition of GSH. For both enzyme preparations, pre-incubation with 1 mM H_2O_2 for 30 minutes inhibited subsequent substrate hydroxylation reactions, inspite of 2 mM ascorbate being freshly added to start the hydroxylation reaction (Fig 4D). Interestingly, further addition of 5 mM GSH could similarly reactivate both enzyme preparations, though reaction kinetics were substantially slower for the reactivated enzymes. (Fig 4D).

Oxygen sensing is fully functional in ascorbate-deficient *Gulo*$^{-/-}$ mice. *Gulo*$^{-/-}$ mice received a diet with or without vitamin C for 5 weeks. Transcript levels of known HIF target genes (Bnip3, Ca9, Glut1, Pdk1, Phd2, Phd3, Ndrg1) as well as genes involved in antioxidative defense (Sod1, Sod2, Glrx), the ascorbate transporters Svct1 and Svct2 or oxygen-independent genes (Ednrb, Mmp3, Phd1) were determined by RT-qPCR in brain, lung, kidney, heart and liver. Similar tissue-specific expression levels for most genes involved in different pathways were observed in both groups (Fig. 5A). Conclusive with our finding from cell culture experiments, expression levels of most of the HIF target genes remained largely unaffected by vitamin C deficiency or even showed reduced levels (blank and blue squares, respectively, in the heatmap shown in Fig. 5B). Expression of the ascorbate transporter Svct1, however, was moderately induced in animals fed without ascorbate, possibly reflecting compensatory mechanisms for ascorbate deprivation.

To further test if the absence of ascorbate limits PHD function under hypoxic conditions, *Gulo*$^{-/-}$ males were deprived from vitamin C for 5 weeks, while control animals were supplemented with vitamin C. As described previously [18], the animals developed a scorbutic phenotype marked by a substantial loss of body weight after 35 days on a ascorbate-free diet, indicating that systemic stores of vitamin C have been exhausted (Fig. 5C, left panel). In line with this observation, plasma ascorbate levels in *Gulo*$^{-/-}$ mice fed an ascorbate-free diet were

below the detection limit (≤1 μM), while plasma of control animals contained 40.3-123.8 μM of vitamin C (Fig. 5C, right panel), corresponding to ascorbate plasma levels in healthy humans [34].

Following 5 weeks of vitamin C deprivation, mice were breathing 8% oxygen for an additional period of 24 hours while control groups were kept under ambient oxygen concentration (see scheme in Fig. 5C). Both ascorbate-deficient and supplemented animals responded to the hypoxic treatment with a robust induction of *Epo* mRNA in the kidney which was higher in *Gulo*$^{-/-}$ males fed without ascorbate, though differences did not reach the level of significance ($p = 0.07$, Student's t-test) (Fig. 5D, left panel). Circulating EPO protein levels in mouse plasma were induced by hypoxic exposure to a similar extent in both groups (Fig. 5C, right panel). Of note, no significant changes of the red cell lineage hematologic parameters were observed in *Gulo*$^{-/-}$ mice after 5 weeks of ascorbate depletion, indicating that the oxygen transport capacity was similar in both treatment groups. Hypoxic increases in hematocrit values are known to be delayed and reach the level of significance not before 72 hours of continuous exposure to hypoxia [35], explaining the lack of an increase of either hematocrit values or red blood cell counts in our experimental setting with a hypoxic period of only 24 hours (Supplementary Table 2).

Discussion

Ascorbic acid and ferrous iron have been reported as essential co-factors for PHD-dependent HIFα hydroxylation *in vitro* [13]. Unexpectedly, we found a fully functional cellular oxygen sensing pathway in HeLa cells maintained under strictly ascorbate-free culture conditions, indicating that ascorbate is dispensable for HIFα hydroxylation *in vivo*. In search for the nature of antioxidative compounds substituting for ascorbate during prolyl-4-hydroxylation, we identified GSH as a potent activator of all three PHDs *in vitro,* increasing HIFα peptide hydroxylation in a dose-dependent manner. Notably, ascorbate and reduced glutathione are the most abundant reducing compounds within eukaryotic cells [36]. Given the distinct antioxidative properties of vitamin C and GSH, the two compounds might affect prolyl-4-hydroxylation by different ways. Ascorbate might be required to reduce occasionally oxidized ferric Fe (III) generated in the active centre of PHDs by uncoupled reaction cycles as it has been described for collagen P4H [27]. However, to the best of our knowledge, no experimental evidence has been reported for enzymatic activity of PHDs in the absence of a hydroxyl-acceptor substrate. Strikingly, the major iron form bound to purified PHD2 is ferrous Fe (II) even when purified under oxygenated conditions [37], arguing against an essential role of ascorbate in reducing PHD iron. In support of this notion, iron and 2-oxoglutarate have been reported to co-purify with 50% and 5-10% of PHD2, respectively, while ascorbate did not co-purify at all [37]. Recent work by Flashman *et al.* showed that ascorbate does not directly interact with the catalytic domain of PHD2, however, its intrinsic ene-diol reducing moiety was found to be important to promote hydroxylation by PHD2 [38].

GSH fully stimulated *in vitro* PHD hydroxylation activity only at rather high concentrations, which is in line with findings previously reported for N-terminally truncated PHD2 [38]. The millimolar GSH concentrations used in our study reflect physiologically relevant levels of this compound like they occur in living cells [39]. Moreover, we found that ascorbic acid is not consumed by coupled substrate hydroxylation, suggesting that exogenously added GSH does not simply regenerate potentially co-purified oxidized dehydroascorbate (DHA). Our data rather favor an alternative function of GSH by preventing oxidative damage to the enzyme itself. Physiological concentrations of GSH were able to reduce transition metal- or peroxide-induced PHD enzyme carbonylation. Despite being generally referred to as antioxidant, ascorbate, together with oxygen and transition metals such as Fe (III) or Cu (II), also exerts pro-oxidative

effects by generating hydroxyl radicals in a Fenton-like reaction [40]. Indeed, we found increased PHD2 carbonylation by ascorbate/iron *in vitro*, suggesting that GSH might protect PHDs from the adverse effects of ascorbate. In line with two distinct reaction modes, addition of GSH to hydroxylation reactions containing saturating ascorbate concentrations markedly increased the hydroxylation rate of PHDs *in vitro*. Of note, Co (II) induced ascorbate depletion, as suggested for cultured cells [28,29], cannot account for PHD inhibition in our cell-free assays, since we previously showed that Co (II) only inefficiently catalyses ascorbate oxidation by air under these assay conditions [16]. Direct interference of Co (II) with the enzymes is supported by the observation that Co (II) strongly carbonylated purified PHD2 even in the complete absence of ascorbate. Moreover, ascorbate and GSH exclusively blunted Co (II)-induced HIF activation in our cellular models, demonstrating a complementary function of GSH and ascorbate in oxygen sensing by living cells. While simple chelation of Co (II) by ascorbate and GSH cannot be fully excluded in cell culture experiments, it should be mentioned that metal chelators naturally occurring in serum (e.g. histidine, glutamic acid, albumin but also GSH) are essential to facilitate Co (II) induced ascorbate oxidation, since "free" Co (II) is unable to directly oxidize ascorbate in simple aqueous solutions at neutral pH (reviewed in [28]). Thus, the actual redox potential of the ion in such ternery complexes - rather than the intracellular concentration of "free" metal - determines its efficacy to act as a "hypoxia mimetic".

One of the specific GSH functions is to prevent or reduce inappropriate disulfide bond formation. Two recent reports identified PHD2 cysteinyl residues C201 and C208 to be highly nucleophilic and surface accessible [32,33]. Moreover, crystallographic analyses predicted that these two cysteines might form disulfide bonds [32,33]. C201S mutation protects recombinant PHD2 from oxidative damage *in vitro* and results in a 2.5-fold higher specific hydroxylation activity. One might speculate that a certain fraction of wild-type PHD2 enzyme constantly undergoes oxidative modification of C201, leading to reduced activity. As such, PHD enzymes could combine oxygen and redox sensing properties, providing a possible explanation for previous work on redox factors modulating PHD activity [41-43]. However, potentiation of PHD activity by GSH clearly involves mechanisms distinct from C201 oxidation, since both, wildtype and C201S mutated PHD2 enzymes could be efficiently reactivated from H_2O_2-induced damage by GSH.

Translating our biochemical and cellular findings in a systemic context, we did not observe marked alterations in HIF target gene expression following dietary vitamin C

deprivation in *Gulo*$^{-/-}$ mice, providing evidence that other antioxidants might substitute for vitamin C *in vivo*. Accordingly, the hypoxic response of *Gulo*$^{-/-}$ mice with low or undetectable ascorbate in the plasma was similar to mice receiving an ascorbate-supplemented diet. Consistent with antioxidant redundancy *in vivo*, a study using the same animals backcrossed to a BALB/c genetic background found vitamin C-independent *de novo* synthesis of collagen in allografted tumors and unchanged levels of hydroxyproline-collagen. Dermal hydroxyproline content of collagen was even increased [44]. Of note, treatment of vitamin C-deprived guinea pigs with a cell permeable GSH monoethyl ester significantly attenuated the severity of scurvy-related symptoms, suggesting a cooperative function of both antioxidants for P4H function also *in vivo* [45]. Moreover, *Gulo*$^{-/-}$ mice have increased levels of total glutathione in brain and liver, possibly explained by a compensatory mechanism for antioxidative defense in these animals [46].

Interestingly, tumor growth and angiogenesis was retarded in a syngenic tumor model using vitamin C-deprived *Gulo*$^{-/-}$ mice, but no changes were observed for HIF-1α protein levels in the respective tumor tissue [47]. The possible value of vitamin C in cancer therapy recently experienced a renaissance, since it has been shown that pharmacological doses of vitamin C decrease growth of tumor xenografts in mice by increasing peroxide levels in neoplastic tissue [30,48]. Such tumoricidal effects of antioxidants might, at least partially, involve destabilization of HIFα following increased hydroxylase activity [49,50]. Our data support a model of cooperative function of GSH and vitamin C in regulating the efficiency of PHD oxygen sensors. Translated to chemotherapy of cancers, combined treatment with both clinically approved molecules might even boost their antitumorigenic function.

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Autorship Contributions

K.J.N. designed and performed experiments, analyzed data, and wrote the manuscript. K.J.N., N.M., Ph.S. and Pa.S. performed *in vivo* experiments. R.H.W designed experiments and wrote the manuscript. D.P.S designed experiments, analyzed data, and wrote the manuscript.

Conflict of Interest Disclosures

The authors declare no competing financial interests.

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Figure legends

Fig 1. Cellular oxygen sensing by the PHD/HIF pathway does not require vitamin C. (A) Proliferation of HeLa cells growing in FCS-free, chemically defined medium containing either no or 50 µM ascorbate. (B) Stabilization of HIF-1α protein in HeLa cells maintained in FCS-free medium containing either no (-Asc.) or 50 µM ascorbate. Cells were exposed to 21 and 0.2% oxygen for 6 hours and protein levels analysed by immunoblotting. (C) Induction of HIF-dependent luciferase activity (pH3SVL vector) in HeLa cells maintained in FCS-free medium containing either no or 50 µM ascorbate and exposed to 0.2%-21 % oxygen for 24 hours.

Fig. 2. GSH substitutes for vitamin C as a co-factor in HIF-α hydroxylation *in vitro*. (A) GSH can enhance PHD hydroxylation activity in the absence of ascorbate (-Asc.) in a dose-dependent manner. Hydroxylation activity was determined using a multi-well VBC binding assay. (B) PHD-dependent hydroxylation reaction rate in the presence of 2 mM ascorbate (control) or 2 mM ascorbate combined with 5 mM GSH (+5 mM GSH). Shown are mean values ± SEM of triplicates. (C) Ascorbate determination by HPLC before and after 1 hour of PHD2-dependent hydroxylation reaction (left panels). Ascorbate content is only slightly decreased after 1 hour of incubation and independent of target hydroxylation (right panel). Shown are mean values ± SEM of three independent experiments normalized to values measured at time point zero.

Fig. 3. GSH impairs HIF activation in cells. (A) Induction of HIF-dependent luciferase reporter gene activity in stably transfected HRG1 hepatoma cells by 2% O_2, 50 µM Dfx or 100 µM $CoCl_2$ for 24 hours. (B) Effects of ascorbate (Asc, 0.2-2 mM; upper panel) or GSH (1-10 mM; lower panel) in combination with hypoxia, Dfx or $CoCl_2$ treatment on HIF-dependent luciferase activity relative to the protein concentration of the lysates. Shown are mean values ± SEM of three independent experiments normalized to the reporter activity in the absence of either ascorbate or GSH (control). (C) CA9 and NDRG1 HIF target gene mRNA levels in HRG1 cells following treatment with 2 mM ascorbate or 10 mM GSH combined with 2% O_2 or 100 µM $CoCl_2$ for 24 hours. Shown are mean values ± SEM of three independent experiments relative to the mRNA content of ribosomal protein L28. (D) HIF-1α and CA9 protein levels in HRG1 cells

following treatment with 2 mM ascorbate or 10 mM GSH combined with 2% O_2 or 100 µM $CoCl_2$. (E) Oxyblot analyses of recombinant PHD2 protein carbonylation. GSH (5 mM) reduced PHD2 carbonylation by either 2 mM ascorbate/10 µM $FeSO_4$ or 100 µM $CoCl_2$ (left panel). PHD2 oxidation is independent of target hydroxylation as shown by using a wild-type or a P564A mutant HIF-1α hydroxyl-proline acceptor peptide in *in vitro* hydroxylation reactions (right panel).

Fig. 4. A C201S mutation enhances PHD2-dependent hydroxylation reaction rate and protects from protein oxidation. (A) Conservation of cysteine residues (C201 and C208 in human PHD2) in all three human (*Hs*) and mouse (*Mm*) PHD isoforms. (B) Increased reaction rate of C201S mutant PHD2 as measured by the hydroxylation-dependent VBC binding assay. Shown are mean values ± SEM of three independent experiments. Linear regression analyses were performed, revealing highly different slopes ($p<0.0001$). (C) The C201S mutation confers resistance of PHD2 to H_2O_2-mediated inhibition of hydroxylation activity. Shown are mean values ± SEM of a representative experiment performed in triplicates. (D) Reduced glutathione (GSH) can rescue PHD2 wt and C201S hydroxylation activities after H_2O_2 -mediated enzyme damage. Briefly, enzyme preparations were pre-incubated with 1 mM H_2O_2 for 30 minutes (H_2O_2) or left untreated for a similar period (control). For rescue experiments, enzymes after H_2O_2 treatment were incubated with 5 mM GSH (H_2O_2+GSH) for 15 minutes. Hydroxylation reactions were carried out at standard assay conditions for 60 minutes. Note that all reactions contained 2 mM ascorbate freshly added when hydroxylation reactions were started. Data is given as mean values ± SEM of three independent experiments normalized to hydroxylation activities of control reactions obtained after 60 minutes.

Fig. 5. The hypoxic response is fully functional in vitamin C depleted *Gulo*$^{-/-}$ mice. *Gulo*$^{-/-}$ male mice received a diet with (+Asc) or without (-Asc) ascorbate for five weeks. (A) Transcript levels of HIF target genes (Bnip3, Ca9, Glut1, Pdk1, Phd2, Phd2, Phd3, Ndrg1) as well as genes involved in antioxidative defense (Sod1, Sod2, Glrx), the ascorbate transporters Svct1 and Svct2 or oxygen-independent genes (Ednrb, Mmp3, Phd1) were quantified by RT-qPCR in brain, kidney, lung, liver and heart. Values are expressed relative to S12 mRNA levels and visualized in a heat map (Genepattern, Broad Institute, USA). Lowest and highest mRNA levels of each

gene were arbitrarily defined as -3 (dark blue) and +3 (dark red), respectively. (B) Heat map of gene expression changes following a vitamin-C-deficient diet. Log_2 (-Asc/+Asc) ratios revealed that the majority of HIF target genes remained either unchanged or showed a slightly reduced expression pattern. Data ranged from -1.75 (dark blue) and +1.79 (dark red), respectively. (C) Scheme depicting the experimental setup for hypoxic experiments with vitamin C depleted *Gulo*$^{-/-}$ animals (upper panel). Relative gain of body weight of *Gulo*$^{-/-}$ mice (n=11 animals per group) following ascorbate withdrawal (-Asc) or ascorbate supplementation (+Asc) for 36 days (left panel). Ascorbate levels in the plasma of *Gulo*$^{-/-}$ mice (n=6 animals per group) after five weeks of ascorbate withdrawal compared to mice kept on an ascorbate-supplemented diet (right panel). (D) Epo mRNA (left panel) and circulating EPO protein (right panel) levels in *Gulo*$^{-/-}$ mice maintained on a diet with (+Asc) or without (-Asc) for five weeks followed by exposure to 8% or 21% oxygen for 24 hours. Data represent mean values ± SEM derived from at least 5 animals per group (n.s., not significant).

Figure 1

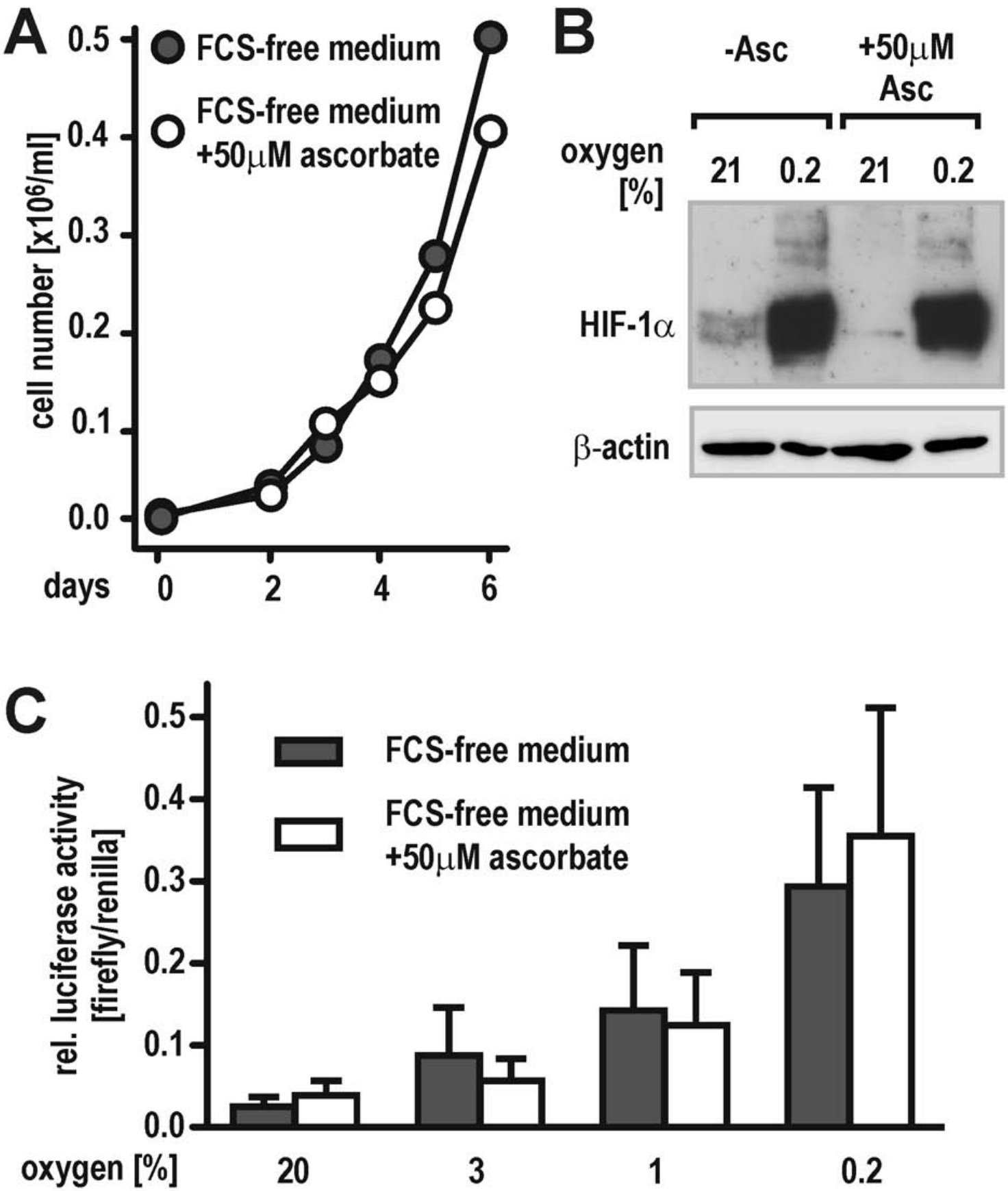

A
0.5
0.4
0.3
0.2
0.1
0.0
cell number [x10⁶/ml]
FCS-free medium
FCS-free medium
+50μM ascorbate
days
0
2
4
6
B
-Asc
+50μM
Asc
oxygen
[%]
21
0.2
21
0.2
HIF-1α
β-actin
C
0.5
0.4
0.3
0.2
0.1
0.0
rel. luciferase activity
[firefly/renilla]
FCS-free medium
FCS-free medium
+50μM ascorbate
oxygen [%]
20
3
1
0.2

Figure 2

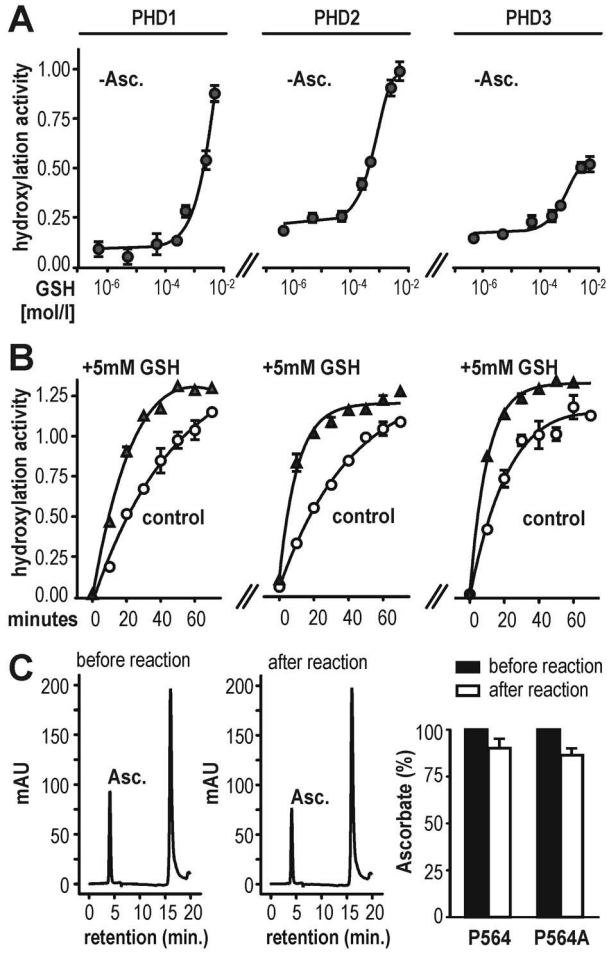

A
PHD1
PHD2
PHD3
-Asc.
-Asc.
-Asc.
hydroxylation activity
1.00
0.75
0.50
0.25
0.00
GSH
[mol/l]
10-6
10-4
10-2
B
+5mM GSH
+5mM GSH
+5mM GSH
control
control
control
hydroxylation activity
1.25
1.00
0.75
0.50
0.25
0.00
minutes
0 20 40 60
C
before reaction
after reaction
mAU
200
150
100
50
0
Asc.
0 5 10 15 20
retention (min.)
before reaction
after reaction
Ascorbate (%)
100
75
50
25
0
P564
P564A

Figure 3

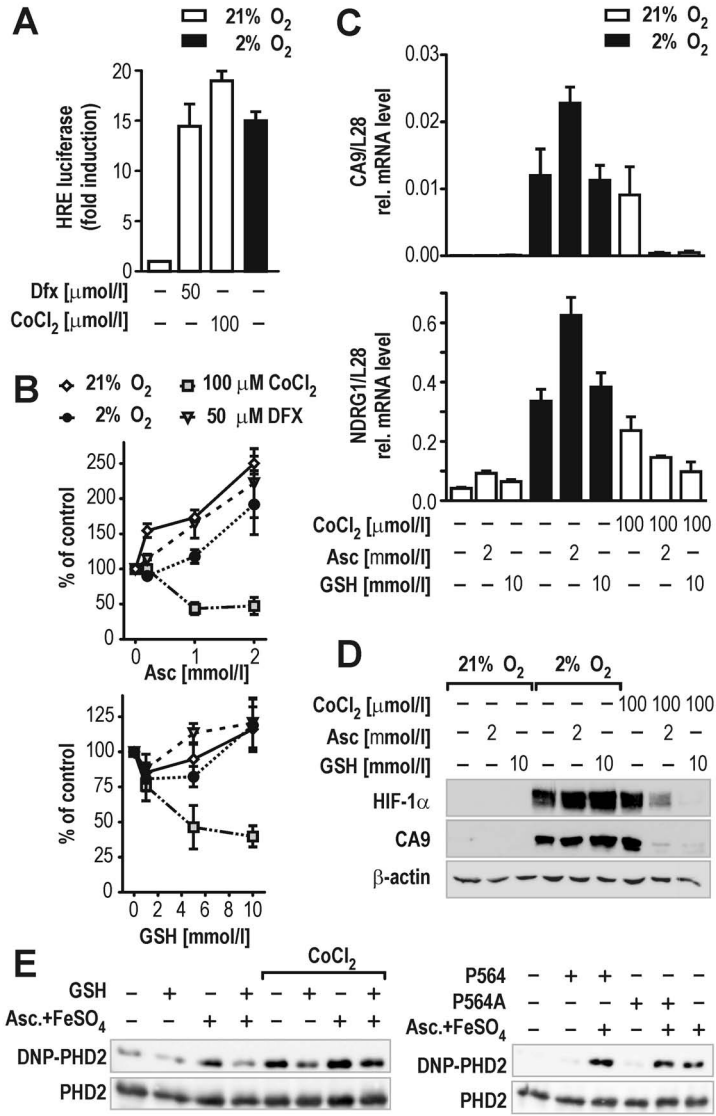

A
21% O2
2% O2
HRE luciferase (fold induction)
Dfx [µmol/l]
CoCl2 [µmol/l]
B
21% O2
2% O2
100 µM CoCl2
50 µM DFX
% of control
Asc [mmol/l]
GSH [mmol/l]
C
21% O2
2% O2
CA9/L28 rel. mRNA level
NDRG1/L28 rel. mRNA level
CoCl2 [µmol/l]
Asc [mmol/l]
GSH [mmol/l]
D
21% O2
2% O2
CoCl2 [µmol/l]
Asc [mmol/l]
GSH [mmol/l]
HIF-1α
CA9
β-actin
E
CoCl2
GSH
Asc.+FeSO4
DNP-PHD2
PHD2
P564
P564A
Asc.+FeSO4
DNP-PHD2
PHD2

Figure 4

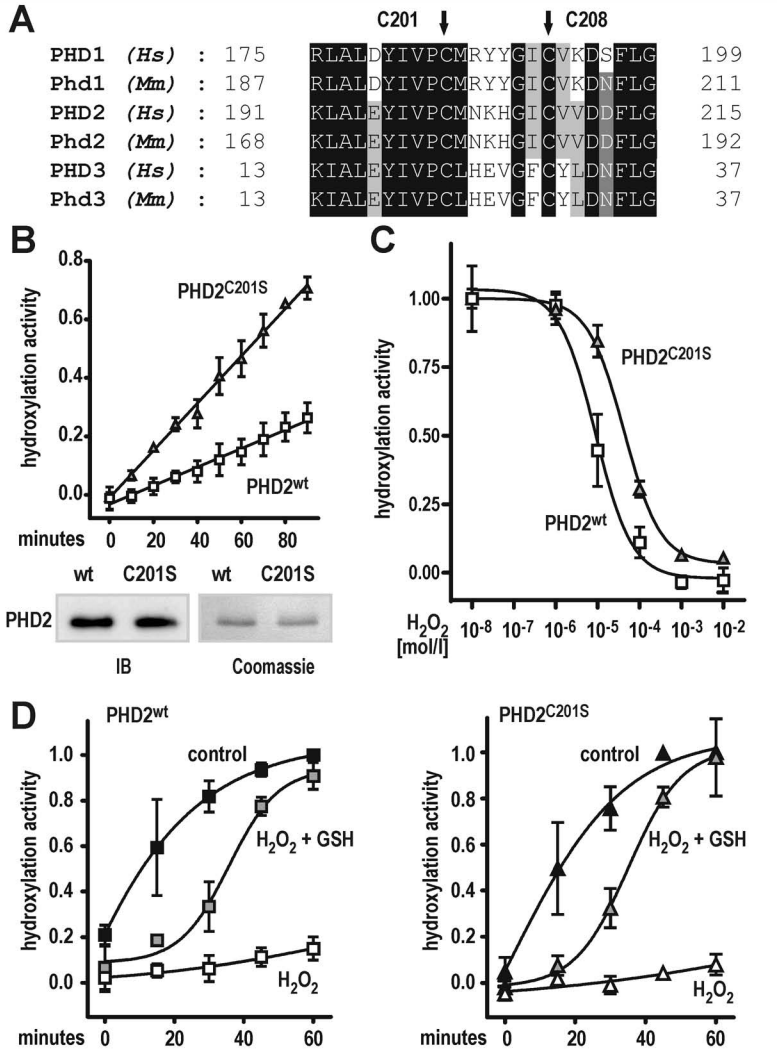
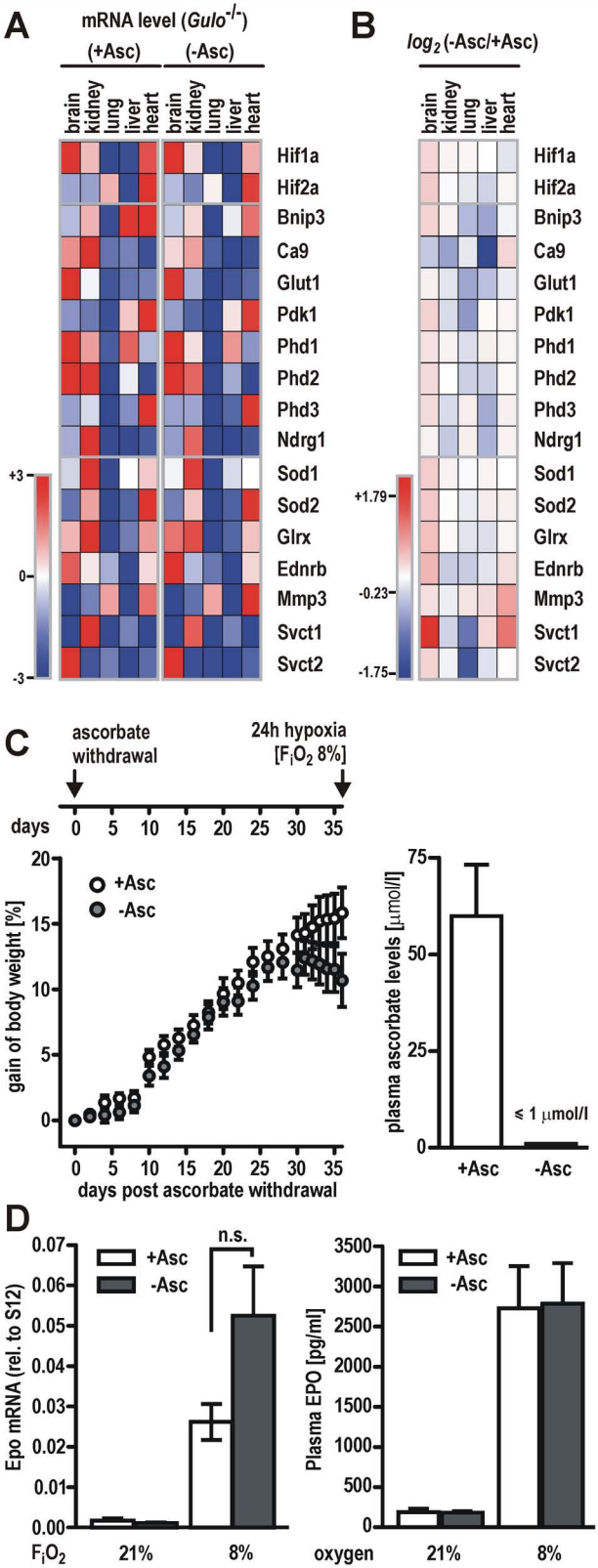

A
C201
C208
PHD1 (Hs) : 175 RLALDYIVPCMRYYGICVKDSFLG 199
Phd1 (Mm) : 187 RLALDYIVPCMRYYGICVKDNFLG 211
PHD2 (Hs) : 191 KLALEYIVPCMNKHGICVVDDFLG 215
Phd2 (Mm) : 168 KLALEYIVPCMNKHGICVVDDFLG 192
PHD3 (Hs) : 13 KIALEYIVPCLHEVGFCYLDNFLG 37
Phd3 (Mm) : 13 KIALEYIVPCLHEVGFCYLDNFLG 37
B
hydroxylation activity
PHD2C201S
PHD2wt
minutes 0 20 40 60 80
wt C201S wt C201S
PHD2
IB
Coomassie
C
hydroxylation activity
PHD2C201S
PHD2wt
H2O2 [mol/l] 10-8 10-7 10-6 10-5 10-4 10-3 10-2
D
PHD2wt
control
H2O2 + GSH
H2O2
hydroxylation activity
minutes 0 20 40 60
PHD2C201S
control
H2O2 + GSH
H2O2
hydroxylation activity
minutes 0 20 40 60

Figure 5


A
mRNA level (Gulo-/-)
(+Asc)
(-Asc)
brain
kidney
lung
liver
heart
Hif1a
Hif2a
Bnip3
Ca9
Glut1
Pdk1
Phd1
Phd2
Phd3
Ndrg1
Sod1
Sod2
Glrx
Ednrb
Mmp3
Svct1
Svct2
+3
0
-3
B
log2 (-Asc/+Asc)
+1.79
-0.23
-1.75
C
ascorbate withdrawal
24h hypoxia [FiO2 8%]
days
gain of body weight [%]
+Asc
-Asc
days post ascorbate withdrawal
plasma ascorbate levels [µmol/l]
≤ 1 µmol/l
D
Epo mRNA (rel. to S12)
n.s.
FiO2
21%
8%
Plasma EPO [pg/ml]
oxygen